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Clostridial Vaccine for Veterinary Use in Iran: A Review

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Authors' contributions

This work was carried out in collaboration among all authors. The first author LAK designed or scope of the review. All other authors contributed to the improvement of the article. All authors read and approved the final manuscript.

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ABSTRACT

Clostridia are Gram-positive anaerobic, spore-forming rods, found in soil, water as well as gastro-intestinal tract of human and animals worldwide. Clostridial infections are among the most prevalent diseases in Iran. *Clostridium* causes botulism, tetanus, food poisoning, wound infections, enterotoxaemia, gas gangrene, necrotic enteritis, pseudomembranous colitis, blackleg and black disease. *Clostridium* also causes several diseases affecting the livestock and poultry industries throughout the world. Vaccination against clostridial infection is effective in immunization of domestic animals and birds. This review discusses clostridial infection and the development of vaccines against their infection in Iran. The last reported outbreaks of blackleg, black disease and enterotoxaemia occurred years ago, so these vaccines have been produced since the 1960s using traditional and conventional methods. In recent years, molecular biology methods have been developed and applied to the identification of clostridial diseases among animals. In this study, molecular cloning strategies for the major toxins of *Clostridium* species, for development of recombinant vaccines, were designed and evaluated. *In vivo* studies indicate that the recombinant vaccines will increase immunity against disease in laboratory animals. These experimental

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vaccines can thus be used in future pilot studies in Iran. This review article presents current knowledge regarding *C. perfringens, C. novyi, C. septicum* and *C. chauvoei* in the veterinary industry in Iran.

Keywords: Clostridium; identification; vaccine; molecular biology.

1. INTRODUCTION

The high rate of clostridial infection cases reported in Iran years ago [1]. These infections causes huge economic losses in livestock and poultry industries. Vaccination against clostridial infection is an effective way in immunization of domestic animals and birds. This review highlights research of development of vaccines in Iran, especially at the Anaerobic Department of the Razi Serum and Vaccine Research Institute.

2. METHODS

In the present review, we conducted a critical research in the literature topics about clostridial infection in Iran using the available International databases (Pubmed, Scopus, Science Direct), and the national database (Iran Medex, SID (Scientific Information Database). We discussed the available vaccines against clostridial infection in Iran, with emphasize on experimental genetically-engineered vaccines providing a better understanding of the new generation vaccine in the future and their importance against clostridial disease in Iran.

3. RESULTS

After research, 77 scientific papers were used for the review. The results showed the situation of clostridial infection and development of vaccine over the years in Iran. The results were grouped and presented as under.

3.1 *Clostridium perfringens*

Clostridium perfringens is a gram-positive, anaerobic, spore-forming, rods, and resistant to environmental conditions. The *C. perfringens* species are classified into several isotypes viz; A, B, C, D, E, F and G based upon the presence of major toxins genes, i.e.iota (*ia*), alpha (*cpa*), beta (*cpb*) and epsilon (*etx*) [2].

3.1.1 Alpha toxin (CPA) or phospholipase C

The gene encoding alpha toxin is located on chromosome of *C. perfringens* types which is translated into an active protein containing 398 amino acids (molecular weight of 43 kDa) [3]. *C. perfringens* type A causes several diseases include food poisoning and gas gangrene in humans, enteritis in animals and necrotic enteritis in chickens [4]. *C. perfringens* type A is the most common type in Iran, North America, Belgium, Korea, Turkey, India and Nigeria [5-7].

3.1.2 Epsilon toxin (ETX)

The gene encoding ETX is located on plasmid of *C. perfringens* type D and B isolates [8]. ETX toxin is secreted as inactive toxin (33 kDa) and activated using intestinal proteases in the intestinal tract of animals which is responsible enterotoxaemia or pulpy kidney in sheep and goats [9].

3.1.3 Beta toxin (CPB)

The gene encoding CPB is located on plasmid of *C. perfringens* type C and B isolates. CPB toxin is secreted as prototoxin (336 amino acids with molecular weight 37kDa) then removed signal peptide resulting in an active form of toxin (approximately 34 kDa) in the intestinal tract of animals. CPB is causes fluid accumulation and necrosis of the intestine with hemorrhage.

3.1.4 Beta2 toxin (CPB2)

The gene encoding beta2 toxin is located on plasmids of *C. perfringens* type B isolates. CPB2 is secreted as a protoxin 265 amino acids and activated in the intestinal tract of animals (active toxin approximately 27 kDa) [10] which is responsible for enteritis in neonatal pigs and diarrhoea in horses [10].

3.1.5 Iota Toxin (ITX)

ITX toxin secreted as inactive toxin by *C. perfringens* type E isolates and activated by proteolytic enzymes in intestinal tract (47 kDa) [11,12]. Iota toxin encoded on a large plasmid, consists of two independent proteins Ia (an enzymatic component), Ib (a binding component) [11] which is responsible for antibiotic-associated enterotoxaemia in lambs and calves [13].

The first strains of *C. perfringens* type D [14] and an Iranian variant of *C. perfringens* type B were isolated from enterotoxaemia cases in 1954 from the country. The Iranian *C. perfringens* variant type B, produces two major lethal toxins (Beta and Epsilon), similar to classical type B strains, but the Iranian strain differs. A classical type B strain produces the minor antigen components of Kappa (Collagenase) toxin instead of Lambda toxin (Proteinase). The Iranian strain is also unable to produce hyaluronidase, which is produced by all classical type B strains [15]. *C. perfringens* type B (classical) causes dysentery in sheep, while the Iranian variant of *C. perfringens* type B causes hemorrhagic enteritis in sheep and goats in Iran. Further studies have found more than 110 toxigenic strains of *C. perfringens* [14] in other diseases, including lamb dysentery, pulpy kidney, malignant oedema, with heavy losses over many years throughout the country. Since 1950, clostridial infections have been studied by Rafiei and Ardehali at the Razi Institute [1].

In 1969, 110 toxigenic strains isolated from the intestinal contents of fish, cattle, and sheep were examined. The most common isolate was *C. perfringens* type A. Also the classical type B strains, Iranian variant of *C. perfringens* type B, and type D were also isolated. However, no strain of *C. perfringens* type C has been isolated in this country prior to 1969.

In 1971, many cases of necrotic enteritis with heavy mortality were observed in Iran in piglets. *C. perfringens* type C was the responsible organism in an outbreak in which 900 out of 1300 piglets died. The first strain of *C. perfringens* type C isolated in Iran from these cases by the Razi Institute [16]. Crude toxoids or bacterin-toxoids vaccines have been effective in domestic animals including cattle, sheep, and goats etc. [17]. In Iran, commercial vaccine is ETX, CPB and CPA bacterin-toxoids.

3.1.6 *C. Perfringens* vaccine production

Because of high mortality rate, vaccination has been done to control of *C. perfringens* diseases. More than 7,000,000 vaccine doses were prepared using papain meat digest medium in 1966. Because of the high demand for enterotoxaemia vaccine and low cost, attempts have been made to use purified horse serum as a culture medium for pulpy kidney vaccine production. The results showed a minimum lethal dose (MLD), which was comparable to that obtained with peptone medium or papain meat digest, which have been used for pulpy kidney vaccines for many years at the Razi vaccine and serum Institute. Therefore, purified horse serum could be used as a nitrogen source for production of enterotoxaemia vaccine [18].

In 1973, a standard preparation plan for *C. perfringens*, *oedematiens*, and *septicum* antitoxins was developed by Ardehali. Sheep were injected with vaccine three times. After seven days of each cycle of injections, sheep were bled and serum was separated. Each sample was purified and concentrated. Antitoxin unit were 1000, 15000, 3000, 250 and 200 IU/ml for *C. perfringens* types A, C, and D, and *C. oedematiens* type B, and *C. septicum*, respectively. Prepared sera were used to identify clostridial infections for years [19].

In Iran, mass production and standardization of the enterotoxaemia vaccine was started in 1976. More than 20 million doses of this vaccine have been manufactured annually [20].

Later, some modifications have been made to improve culture media. Potency of the vaccine was estimated in sheep and rabbits. The result of this study was satisfactory. Also, no cases of lamb dysentery and pulpy kidney diseases was reported from the field since immunization [21].

Because of the more enhanced demand for the vaccine, a new plan was developed by Ardehali in 1979, in the anaerobic department of the Razi Institute. At first, *C. perfringens* types B, C, and D, and *C. oedematiens* vaccines were cultured separately. Then samples were collected for MLD determination in N.M.R.I mice model. Animals were injected with vaccine containing adjuvant (potash alum) and anaculture. Levels of antitoxin were estimated in the animals. All injected animals showed high-titer of antibodies [22].

An enterotoxaemia vaccine has been prepared in the traditional manner (bottle glass) at the Razi Institute for four decades until 1989. Since 1990, the enterotoxaemia vaccine has been produced in a fermenter (unpublished data).

3.1.7 Study of a recombinant vaccine

Nowadays, researches have been focused on new generation vaccines and several

experimental monovalent recombinant *C. perfringens* vaccines have been prepared. In 2010, Taherian Fard and colleagues extracted the CPE C-terminal region from *C. perfringens* type A strain, and cloned it into the pET32a vector. This study showed that purified C-CPE had not had any systemic effect on laboratory animals [23].

Bioinformatics have been conducted on the fusion of the epsilon and beta toxin genes of *C. perfringens* type D and B, introduced recent technological developments in design of the hybrid toxin structure. The *etx* and *cpb* genes were obtained from Gen Bank were used to design a chimeric fusion gene. The secondary and tertiary structure characteristics of this fusion protein were predicted using online software. Also, locations for a linker fragment and transmembrane helices were predicted using bioinformatics software. The results showed that the designed fusion gene could be expressed in host cells [24,25].

Pilehchian developed a new strategy for cloning and production of experimental recombinant vaccine of *C. perfringens* beta toxin. The result of *in vitro* and *in vivo* studies were successful [26,27].

The same process was performed in production of recombinant vaccine from the *C. perfringens* epsilon toxin [28,29]. Also, recombinant bivalent vaccines have been prepared and compared to the traditional multivalent vaccines. The result showed amounts of antitoxin were 6 and 10 IU/ml for epsilon and beta, respectively. As a result, *E. coli* was proposed as an appropriate host for expressing the fusion of *C. perfringens* epsilon and beta toxins [30].

In another study, Alimolaei designed a non-toxic mutated ε-toxin gene. This mutated ε-toxin gene lacking toxicity was synthesized and cloned in *Lactobacillus casei*. This study showed that LC-pT1NX-ε could be a promising candidate vaccine against enterotoxaemia [31].

Experimental monovalent recombinant Iota and TpeL toxins have been cloned and expressed in *E. coli* successfully [32,33]. On the other hand, bivalent recombinant epsilon-alpha fusion protein have expressed in *E. coli* successfully [34]. Furthermore, experimental trivalent recombinant CPA, NetB and TpeL resulted in high immunity against disease [35].

3.2 *Clostridium novyi*

C. novyi or *C. oedematiens* is a Gram-positive rod, spore-forming and strictly anaerobic [36]. *C. novyi* is classified into several isotypes, A, B, C and D. *C. novyi* type B and *C. novyi* type type A produces alpha toxin (TcnA), with glycosyl transferase activity [37]. The gene encoding TcnA (molecular weight 250–300 kDa) is located on prophage genome in *C. novyi* isolates [38]. Furthermore,*C. novyi* type B produces beta toxin with phospholipase activity [39] which causes black disease or infectious necrotic hepatitis in sheep and cattle herd [40].

The first strain of *C. oedematiens* type B was isolated from liver lesions of sheep with black disease in 1969. These strains were isolated and identified using fluorescent-labeled antibodies. Isolates were typed according to lecithinase production, haemolytic activity, necrotizing activity, and lethality. Of 33 *C. oedematiens* isolates from all over the country, 27 were identified as *C. oedematiens* type B. Some of these were very toxic, so these were used to produce of black disease vaccine. Three strains of *C. oedematiens* type A were isolated from liver lesions of sheep with black disease and one strain of *C. oedematiens* type D, which causes bacillus hemoglobinuria in cattle was also isolated. These strains were neither pathogenic nor toxic in laboratory animals. Some of the isolates were strongly hemolytic in red blood cells of rabbit, sheep, cattle, and horses [41]. The economic losses can be inhibited by *C. novyi* vaccines in animals [42].

3.2.1 *C. novyi* vaccine production

Firstly, a black disease vaccine was produced as a clostridial combined vaccine (*C. perfringens* types B, C, and D, and *C. oedematiens*). Later, the monovalent black disease vaccine was produced and standard (unpublished data).

Until 1977, the black disease vaccine was produced using digested meat. Since 1978 it has been developed and formulated using peptone, maltose, L. cysteine, and NaOH (unpublished data).

Because of the high demand of the country for vaccination from 1981 to 1982, *C. oedematiens* type B strains were isolated from liver lesions,

and due to heavy economic losses in different parts of the country, a plan was developed in the anaerobic department of the Razi Institute for preparation and standardization *C. oedematiens* vaccine again. This plan was including concentrated toxin preparation, production of standard antitoxin, and effective and safe vaccine preparation. The prepared vaccine was highly immunogenic and was validated by laboratory tests according to the British Pharmacopoeia Standard and field reports [43].

Another large scale effort was made for preparation an effective vaccine against black disease. The prepared vaccine was concentrated and alum potassium (adjuvant) was added and a potency test was carried out in pooled rabbit serum. Amounts of alpha antitoxin were estimated higher than the international standard. Reports in the field indicated that black disease was controlled in sheep [44,45]. Now a days, producers change some elements in order to Induction of toxin production and reduction of time of cultivation of *Clostridium novyi* by growing vaccine strain in fermenters containing previous element plus vitamins and trace element, tween 80 and glycerol [46].

3.2.2 Nano-toxoid vaccine

Experimental pentavalent toxoid vaccine containing *C. perfringens* types D, C, and B, *C. septicum*, as well as *C. novyi* have been prepared and the chitosan was added to pentavalent vaccine then evaluated using *in vivo* and *in vitro* assay. The result showed chitosan vaccine could be used to induce of immunity higher than the toxoid vaccine [47].

3.2.3 Study of a recombinant vaccine

Bioinformatics studies were conducted on *C. novyi* alpha toxin in order to introduce recent technological developments in design of recombinant toxin structure. The result of study showed even small fragment of alpha toxin can produce immune responses [48]. Also, Insilco analysis was conducted on the alpha toxin of *C. novyi* and epsilon toxin of *C. perfringens* in order that design a fusion protein. The result of study showed fusion protein can be used to induce of immunity faster, cheaper and more effective than conventional vaccine [49]. Experimental monovalent recombinant vaccines the alpha toxin of *C. novyi* has been prepared and evaluated using *in vivo* and *in vitro* assay. The results of study showed antibody produced had higher affinity than normal toxin [50,51]. This result could serve as a model for development and production of recombinant vaccines [50].

3.3 *Clostridium chauvoei*

Clostridium chauvoei is a Gram positive rod, spore-forming and strict anaerobic. *Clostridium chauvoei* toxin A (CctA) (molecular mass 32 kDa) is causes of cell lysis [52]. Other virulence factors are including sialidase, beta toxin-DNAse, hemolysin, hyaluronidase, and flagella [53]. *C. chauvoei* is responsible for blackleg in cattle and rarely sheep or small ruminants [54]. Disease is progressive in a short time and kills animals [54].

The first strain of *C. chauvoei* was isolated from cases of cattle with blackleg in 1938 [55].

Blackleg mostly occurs among animals on enzootic farms, but one extensive outbreak of blackleg occurred in August 1968 among cattle in two southern provinces of Iran (Fars and Khuzestan). In this outbreak, more than 400 cattle died [56,57]. A large number of *C. chauvoei* strains, have been detected and isolated from blackleg cases in different parts of Iran for several years. Vaccination with whole formalin-inactivated bacterial cultures is effective way for protective against blackleg disease [8]. Commercially *C. chauvoei* vaccines are monovalent or in combination with other *Clostridium* strains.

3.3.1 *C. chauvoei* vaccine production

Blackleg vaccine has been produced since 1950 for immunization of cows in the country. The prepared vaccine was immunogenic, and safe in injected animals [58].

In 1992, attempts were made to produce and formulate aluminum hydroxide gel plus blackleg anaculture to obtain a highly immunogenic vaccine at the Razi Institute using a traditional procedure to immunize cattle against blackleg disease. Safety and potency tests were carried out in target and laboratory animals. The result of this study showed vaccine was effective against blackleg disease in animals [59].

In 2002, a blackleg anaculture vaccine was produced in large scale, because of the high demand, using fermenter in the traditional manner [60]. Blackleg vaccine has been

prepared in the traditional manner at the Razi Institute for four decades.

In 2007, a concentrated blackleg vaccine was prepared in fermenter to increase of the immunogenicity of vaccine. This vaccine was concentrated by precipitation, and Merthiolate and aluminum hydroxide was added as preservative and gel adjuvant, respectively. The result was satisfactory in guinea pigs [61].

Because of the reduce cost, the potency of the prepared reduced dose, concentrated blackleg vaccine was evaluated in according to Pharmacopoeia as follows. Cattle and guinea pigs were injected twice with 2 ml of the concentrated experimental vaccine, instead of 3ml of the routine vaccine at intervals of two weeks. When challenged, inoculated animals were immune to the challenge dose [62].

In another study, efforts were made to evaluate a modified blackleg and hemorrhagic septicemia vaccine. *Pasteurella* cells were removed using alum treatment and replaced with *C. chauvoei* anaculture. Safety tests were performed in mice, rabbits, guinea pigs, sheep, and cattle. Anaphylactic shock and local inflammation were not observed in laboratory animals or target animals. The modified vaccine was potent and efficient against challenge with *C. chauvoei* in guinea pigs [63,64].

Due to increased demand of the country in 2012, the decision was made to increase of the production procedure of blackleg vaccine using enriched culture medium in a fermenter. The use of enriched medium increased the cell count of *C. chauvoei*, significantly higher than using the conventional procedure in glass bottles. The safety and potency of the improved vaccine was performed in sheep and guinea pigs. Due to the satisfactory result, blackleg vaccine has since been produced using enriched culture medium in fermenters [65].

The efficacy of the blackleg vaccine was surveyed in 394 vaccinated cattle and 1519 unvaccinated cattle (control group) from 2004 to 2006. The result of this study showed no cases of disease were found in the vaccinated cattle, while some cattle in the unvaccinated cattle died or became ill. The difference between vaccinated and unvaccinated animals was significant ($P< 0.05$). This result showed vaccination can protect against blackleg disease [66].

3.4 *Clostridium septicum*

Clostridium septicum is a gram positive spore-forming rod, obligate anaerobic. Alpha toxin (ATX), a pore-forming toxin, is one of the major virulence factors. The gene encoding alpha toxin is located on chromosome of *C. septipum* encoded by the *csa* gene. Alpha toxin secreted as an inactive protoxin containing 443 aa and activated using proteolysis enzyme (molecular weight 46kDa) [67]. *C. septicum* alpha toxin responsible for myonecrosis [68] in man. *C. septicum* is causes severe economic losses in livestock and poultry industries. *C. septicum* is responsible for braxy in sheep and cattle [69], malignant edema (gas gangrene) in ruminants [70] and Clostridial dermatitis in birds [71]. In 1969, three strains of *C. septicum* were isolated from malignant oedema in cattle in Iran. Based on biochemical properties and pathogenicity in guinea pigs and cattle, these isolates were identified as *C. septicum* [14]. The toxins produced by these isolates were completely neutralized and confirmed by *C. septicum* antiserum [14]. Vaccination with bacterin-toxoid preparations is effective way for protective against *C. septicum* disease [72].

3.4.1 *C. septicum* vaccine production

The vaccine produced by growth of *C. septicum* vaccine strains in a fermenter using media such as meat peptones, tryptone, yeast extract, casein hydrolysate, glucose, trace elements and vitamins, L-cysteine hydrochloride and NaOH and inactivation by adding formaldehyde. Experiments were carried out to determine minimum lethal dose, sterility, residual toxicity, safety, and potency. Amount of alpha antitoxin *C. septicum* was higher than the international standard. The results showed that the experimental vaccine was effective and safe [73]. Recombinant experimental vaccine was made against braxy disease in Iran.

3.4.2 Study of a recombinant vaccine

The alpha toxin of the *C. septicum* vaccine strain was cloned in TOP10 *E. coli* cells [74]. In 2015, Insilco analysis has been conducted on the fusion of the epsilon toxin *C. perfringens* type D and alpha toxins *C. septicum* [75]. *The* results showed the designed fusion structure is suitable construction so, this epsilon-alpha fusion gene was developed in 2018 [76]. Also, bioinformatics analysis has been conducted on the chimeric fusion of the alpha toxin genes of *C. septicum*

and *C. perfringens* type A introduced it as candidate vaccine against clostridial disease [77]. This alpha-alpha chimeric fusion gene could be used for the development of a recombinant vaccine [77].

4. DISCUSSION AND CONCLUSION

Worldwide varieties of clostridial vaccines have been used to protect animals. Some of clostridial vaccines have not been used at all in Iran including tetanus toxoid and *C. haemolyticum* for protection of animals. Also, in some countries, a polyvalent vaccine consisting of *C. septicum*, *C. novyi, C. chauvoei,* and *C. sordellii* is used while; polyvalent enterotoxaemia consisting of *C. septicum* and *C. perfringens* is available in Iran. In Iran, a monovalent blackleg vaccine is available, which consisting killed strain of *C. chauvoei*. No cases of some clostridial strain have been reported so far. In Iran, an Iranian variant of *C. perfringens* type B has been found which expresses different minor antigens from classical type B strains. For more than half a century, many attempts have been made to produce clostridial vaccines in Iran. Briefly, several vaccines have been commercialized or are being developed as an experimental vaccine in Iran. Each vaccine with any technology has advantages and disadvantages for various reasons, including efficacy, injection dose, amount of injection, stability in environmental conditions and induction time of the immune system, etc., which is available as a commercial vaccine in the country. Perhaps, the success of recombinant vaccine has the potentials the production of low-cost vaccines with their high efficacy and safety in the future. Further improvements on new generation vaccine could be achieved by genetically or engineered modify improvement. Better understanding of production process would facilitate control of clostridial disease.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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